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ORIGINAL ARTICLE

Gibberellins and light synergistically promote somatic embryogenesis from the in vitro apical root sections of spinach

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Abstract

Gibberellins (GAs) play a pivotal role in the induction of somatic embryogenesis from in vitro root apices of spinach plants. With the aim to understand the role of GAs in this process and to improve somatic embryo (SE) regeneration efficiency, the impact of light and GAs on SE initiation from the in vitro root apices was studied. The root sections were isolated from in vitro-grown SE-derived plants and placed on medium containing 20 µM α-naphthaleneacetic acid (NAA) and 0–10 µM GA_3 or GA_1, and cultivated under light conditions or in darkness. The most efficient SE regeneration response (100% regenerating SEs and 40.73 SEs per root apices) was achieved only in the presence of both light and GAs, with GA_3 always exhibiting much stronger effect than GA_1. Considering that light enhances GAs biosynthesis and the necessity of GAs for SE initiation, the expression levels of genes encoding the key enzymes involved in the final steps of GAs synthesis (*SoGA20-ox1* and *SoGA3-ox1*) and deactivation (*SoGA2-ox1*, *SoGA2-ox2* and *SoGA2-ox3*) were analyzed. Light enhanced the expression of all five *GA-ox* genes, while exogenously supplied NAA + GA_3 provoked downregulation of *SoGA20-ox1* and *SoGA3-ox1* and upregulation of *SoGA2ox-2* and *SoGA2ox-3* expression. The expression of *SoGA2ox-1* only slightly decreased. The results indicated the capability of isolated spinach roots to perceive the light and autonomously produce GAs. The expression levels of genes encoding key enzymes involved in GA biosynthesis suggest that lower levels of GAs favor SE initiation.

Key message

Light and gibberellins (GA) synergistically promote somatic embryogenesis in spinach.Expression levels of genes encoding key enzymes for GA metabolism suggest that lowerlevels of GAs may favor somatic embryogenesis.

Keywords Gibberellin · Light · Regeneration · Root sections · Somatic embryogenesis · Spinach

Introduction

Spinach (*Spinacia oleracea* L.) is a worldwide-cultivated, economically important, green leafy vegetable, rich in nutrients and low in calories. Spinach leaves are a rich source of minerals, vitamins, phytochemicals and dietary fibers

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(Shohag et al. 2011; Kim et al. 2016; Roberts and Moreau 2016). Aqueous extracts of spinach leaves exhibit powerful antioxidant activity, comparable to that of known superior antioxidants, such as green tea and vitamin E (Lomnitski et al. 2003). A spinach-supplemented diet decreases the onset of age-related declines in cognitive and motor functions of the nervous system, and exerts numerous health-promoting effects (Joseph et al. 1999; Lomnitski et al. 2003; Kim et al. 2016; Roberts and Moreau 2016). Therefore, it is not surprising that the production and consumption of spinach plants increased 10 times over the past four decades (http://faostat3.fao.org/browse/Q/QC/E).

Crop improvement programs through genetic engineering require an efficient in vitro regeneration system. However, spinach has long been considered as a plant species recalcitrant to *de novo* regeneration, and regeneration protocols were developed only at the end of the twentieth century. Somatic embryogenesis, as a dependable method of in vitro plant propagation, was achieved in spinach with the highest efficiency using root fragments as the initial explants (Komai et al. 1996a; Ishizaki et al. 2001; Milojević et al. 2011; Nguyen et al. 2013). The absolute necessity of exogenous gibberellic acid (GA_3) for efficient induction of somatic embryogenesis has been recognized in all these studies (Komai et al. 1996a, b; Knoll et al. 1997; Ishizaki et al. 2001; Nguyen et al. 2013).

Besides the content of plant growth regulators (PGRs) in cultivation media, environmental factors, especially light, also play an important role in the induction of de novo regeneration. Responses of the explants to light during somatic embryo (SE) initiation may be species-, variety- or even explant- dependent (Economou and Read 1987). In some plant species, like common bean and *Petunia hybrida*, light was essential for the induction of de novo regeneration (Reuveni and Evenor 2007; Cabrera-Ponce et al. 2015), while in others dark pre-treatment of the explants was necessary for subsequent plant regeneration (Kanwar et al. 2010; Nameth et al. 2013; Muktadir et al. 2016; Talla et al. 2018).

It is well known that long-day (LD) conditions promote elongation of rosette plants, such as spinach, due to enhanced gibberellin (GA) biosynthesis (Zeevaart et al. 1993; Talon et al. 1991; Blazquez et al. 2009). The final steps of GA biosynthesis include the successive oxidation of GA_{53} to GA_{20}, catalysed by GA20-oxidase (GA20-ox). These enzymatic reactions are enhanced when spinach plants are exposed to LD conditions (Wu et al. 1996) due to up-regulation of the *SoGA20-ox1* gene expression (Lee and Zeevaart 2002). In the next step, GA3-oxidase (GA3-ox) converts GA_{20} to GA_1, which is active per se in spinach (Talon et al. 1991; Graebe 1987). Finally, the deactivation of bioactive GAs is catalyzed by GA2-oxidase (GA2-ox). Therefore, the activities of GA20-ox, GA3-ox and GA2-ox directly regulate the levels of active GAs (Hedden and Phillips 2000; Yamaguchi and Kamiya 2000).

Previous studies on the influence of photoperiod and light intensity on spinach regeneration capacity produced contradictory results (Geekiyanage et al. 2006; Milojević et al. 2012). While Geekiyanage et al. (2006) showed that the regeneration of buds from spinach cotyledons was more effective under short-day (SD) than under LD conditions, somatic embryogenesis from the apical root segments of spinach was undoubtedly more efficient under LD than under SD conditions (Milojević et al. 2012; Milić et al. 2017).

Considering the importance of light for GA biosynthesis and the necessity of GAs for SE initiation in spinach, the aim of the present study was to analyze the effects of exogenous GAs (GA_1 and GA_3) and light on SE-forming capacity of spinach roots. In order to elucidate the role and interaction of light and GAs in the promotion of somatic embryogenesis in spinach, we analysed the expression levels of genes encoding the key enzymes involved in final steps of bioactive GA synthesis and deactivation (*SoGA20-ox1*, *SoGA3-ox1*, *SoGA2-ox1*, *SoGA2-ox2* and *SoGA2-ox3*) in the explants grown under inductive and noninductive conditions.

Materials and methods

Basal media

The basal medium (BM) contained full strength macro and micro salts (Lachner, Brno, Czech Republic) according to Murashige and Skoog (1962), 20 g/l sucrose, 100 mg/l myo-inositol, 2 mg/l thiamine, 2 mg/l pyridoxine, 5 mg/l nicotinic acid and 2 mg/l adenine (Sigma Aldrich, St. Louis, MO, USA). Media were gelled with 0.7% agar (Torlak Institute, Belgrade, Serbia) and sterilized at 114 °C and 80 kPa for 25 min. Before sterilization the pH was adjusted to 5.5 using a pH meter.

Plant material

Regeneration of SEs was induced from 1 cm-long apical root fragments, isolated from SE-derived plants of spinach cultivar Matador. Plants of two single seed-derived lines, 125 and 238-6-3-1, obtained by somatic embryogenesis in previous studies, were used as donor plants for this study (Milojević et al. 2011, 2012). The donor plants were maintained on BM supplemented with 5 µM furfurylamino purine (kinetin, Kin, Sigma-Aldrich) under SD light conditions (8 h light and 16 h dark), to prevent precocious flowering of the plants.

The influence of exogenous gibberellins and light on SE-forming capacity of the explants

To assess the impact of GAs and light on SE induction, the root apices were cultivated on regeneration media comprising BM supplemented with 20 µM αnaphthaleneacetic acid (NAA, Sigma-Aldrich) and 0, 2.5, 5, 7.5, 10 µM GA_3 or GA_1, under LD conditions (16 h light and 8 h dark) or in darkness. GA_3 was purchased from SigmaAldrich, and GA_1 was a kind gift from Dr. R.P. Pharis, University of Calgary, Alberta, Canada. GA_3 and GA_1 were dissolved in absolute ethanol, filter sterilized (0.22 µm, Merck Millipore, Billerica, MA, USA) and added to the sterilized medium cooled to approximately 40 °C.

Under LD the root apices cultures were maintained under diffuse light provided by cool white fluorescent tubes (Tesla, Belgrade, Serbia), with a photosynthetic photon flux density of 100 µmol m^2 s^{-1} as measured by an LI-1400 DataLogger equipped with an LI190SA Quantum sensor, LICOR Biosciences. All cultures were cultivated at 25 ± 2 °C.

Each treatment lasted for 12 weeks, with subcultures at 4 week intervals. The number of SEs produced per root apex was recorded at the end of each subculture. SEs were then transferred to BM supplemented with 5 µM Kin for further development and multiplication, while the remaining root apices' tissue was transferred to fresh regeneration medium of the same composition. A trifactorial experiment was set up for the following factors: GA type (GA_3 or GA_1), GA concentration (0, 2.5, 5, 7.5 or 10 µM), and light conditions (LD or dark). Each treatment consisted of four replications (Petri dishes) containing five subsamples (one cm root apices) each, for a total of 20 root apices per treatment. All treatments were arranged in a completely randomized design. The obtained results are presented as the frequency of roots regenerating SEs, the mean SE number per in vitro root apex, and an index of somatic embryo forming capacity (SEFC), calculated as follows: SEFC = (mean SE number per root) × (% of roots with SEs)/100. All these parameters were calculated for the overall 12-week period.

Histological analysis

The apical root fragments, cultivated on BM + 20 µM NAA + 5 µM GA_3 regeneration medium under LD conditions for 4 weeks, were sampled for histological observation in order to explore the origin of SEs regenerated from the in vitro roots. Samples were fixed with FAA (formalin:acetic acid:ethanol = 10:5:85) at 4 °C, dehydrated in a graded ethanol series and embedded in paraffin wax at 58 °C. Sections (7 µm thick) were stained with Toluidine Blue (Sakai 1973) or double-stained with Alcian blue/PAS technique (McManus and Mowry 1960), and photographed using a Zeiss Axiovert microscope (Carl Zeiss GmbH, Gottingen, Germany).

Expression of the GA-ox genes in the root explants

Expression levels of the *GA-ox* genes (*SoGA20-ox1, SoGA3-ox1*, *SoGA2-ox1*, *SoGA2-ox2* and *SoGA2-ox3*), encoding key enzymes for GA metabolism, were analysed using quantitative real time RT-qPCR method. One-cm-long root apices isolated from randomly chosen seedlings were used for gene expression analysis. Equal number of root apices (1–3) excised from one seedling were used for each treatment, thus root apices of the same seedlings were used for all treatments. At least 20 seedlings were used per biological repetition (one RNA sample). The experiment was repeated three times, each with three replicates. For RNA isolation, the root apices were cultivated under LD conditions or in the dark for 4 weeks. The root apices cultivated under LD were collected for RNA isolation 4 h after switching on the light.

To assess the influence of light on the expression of *GA-ox* genes, the explants were cultivated on PGR-free medium under LD conditions, while the explants cultivated on the same medium in the dark were used as a control.

To analyze the influence of exogenous GA on the expression of *GA-ox* genes, the explants were cultivated on regeneration induction medium containing BM + 20 µM NAA + 5 µM GA_3, under LD conditions, whereas the explants grown on PGR-free medium under LD conditions were used as a control.

Total RNA from each sample was extracted from 150 mg of root tissue according to the procedure of Gašić et al. (2004). To remove genomic DNA contamination, samples were treated with DNaseI (Thermo Scientific, Waltham, MA, USA) at 37 °C for 30 min, according to the manufacturer's instructions. First strand cDNA was synthesized in a 20 µl-reaction mixture containing 1 µg of total RNA, using High-Capacity cDNA Reverse Transcription Kit (Life Technologies).

RT-qPCR was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The 10 µl reaction mixture contained Maxima SYBR Green/Rox qPCR Master Mix (Thermo Scientific), 300 nM primers and 1 µl cDNA template. Gene specific primers for *SoGA20-ox1* (GenBank™ accession number U3330, Wu et al. 1996), *SoGA3-ox1*, *SoGA2-ox1*, *SoGA2-ox2* (GenBank™ Accession Numbers AF506280.1, AF506281.1 and AF506282.1, respectively, Lee and Zeevaart 2002) and *SoGA2-ox3* (GenBank™ Accession Number AY935713.1, Lee and Zeevaart 2005), were designed using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast; Ye et al. 2012) (Supplementary Table S1). The amplification protocol included: initial denaturation at 95 °C for 5 min, followed by 36 cycles of

denaturation at 95 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 1 min.

Expression of all tested genes was normalized to the expression of *αTUBULIN* (*α-TUB*, GenBank™ accession number M21414.1, Kawade and Masuda 2009) and calculated relative to the expression of the appropriate control according to the ΔΔCt method (Livak and Schmittgen 2001). Primers used for amplification of *α-TUB*-specific sequence are given in Supplementary Table S1. The obtained values were subjected to $\log_2$ transformation and presented as relative expression values (REV).

Statistical analysis

For SE regeneration response, percentage data were subjected to angular transformation and SE number data were subjected to square root transformation prior to analysis, followed by inverse transformation for presentation. Statistical significance between treatments was tested by standard analysis of variance (ANOVA). The means were separated using Fisher's LSD post hoc test for $p \leq 0.05$. Statistical significance of gene expression data was estimated using t-test for dependent samples.

Results

Influence of exogenous GAs and light on regeneration capacity of the explants

Both light and GAs exerted a significant impact on SE initiation from spinach root apices. The frequency of the roots regenerating SEs, the mean SE number per root apex and the index of SEFC were all significantly influenced by both factors (Supplementary Tables S2 and S3).

NAA, which was present in all media, could only seldom induce SEs as a sole PGR. Out of 9 lines tested in a preliminary study, only the root apices of lines 125 and 238-6-3-1 were capable of SEs regeneration in the absence of exogenous GAs under light. Therefore, these two lines were chosen to study the effect of light and GAs on SE regeneration from the root explants.

In the absence of both GA and light, only the root apices of line 125 were able to regenerate SEs, with frequency of 0.95% (Fig. 1a), 0.14 SEs per root apex (Fig. 1b) and the index of SEFC = 0.05 (Fig. 1c). In the presence of light and in the absence of GA, regeneration capacity slightly improved in line 125, with the frequency of regeneration of 52.03% (Fig. 1a), an average of 8.85 SEs per root apex (Fig. 1b) and the SEFC index of 4.60 (Fig. 1c). Under these conditions, line 238-6-3-1 exhibited significantly lower regeneration capacity than line 125 with the

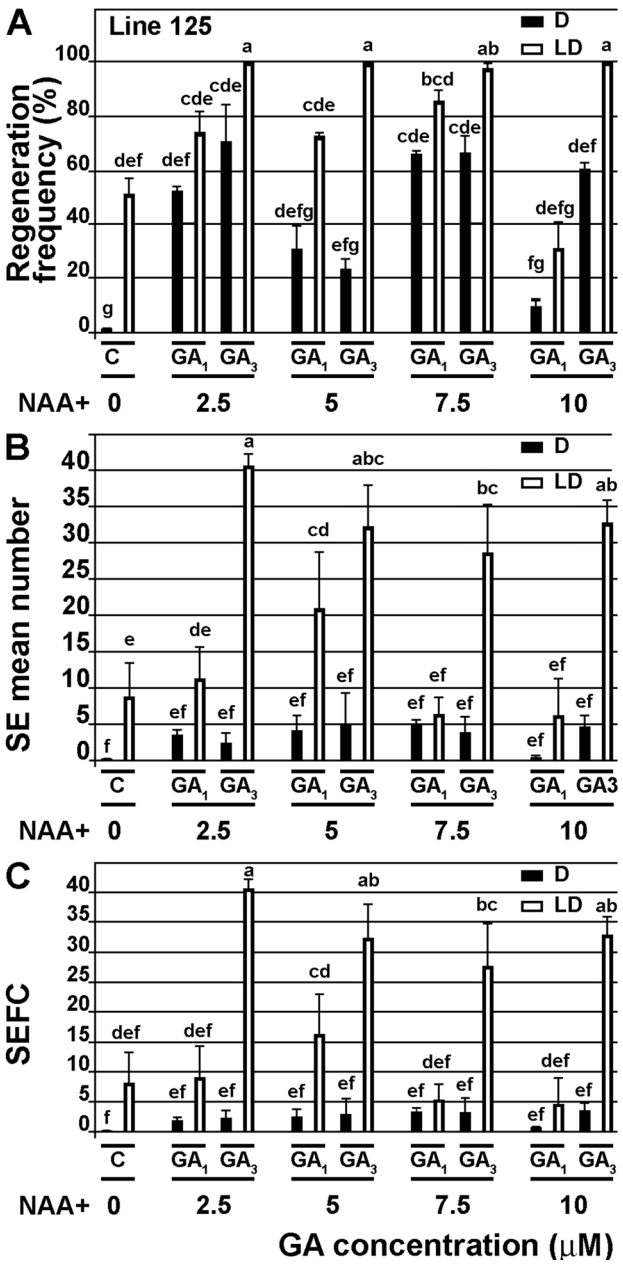

Fig. 1 Somatic embryogenesis from in vitro root apices isolated from SE-derived plants of line 125. The root explants were cultivated on BM supplemented with 20 μM NAA as a sole PGR (control – C) or in combination with 2.5, 5, 7.5, 10 μM GA_1 or GA_3 under long day (LD) or dark (D) conditions for 12 weeks. **a** Frequency of regeneration. **b** The mean SE number per explant. **c** Somatic embryo-forming capacity (SEFC). Data represent the mean values. For each treatment, four Petri dishes, each with 5 root apices (n = 20), were used. The values marked with the same letters are not statistically significantly different ($p \leq 0.05$) according to the LSD test

frequency of 19.17% (Fig. 2a), 2.17 SEs per root apex (Fig. 2b) and SEFC = 1.91 (Fig. 2c).

In the presence of light and after addition of GA_3 to the BM, the root apices of line 125 regenerated SEs with

the frequency of 100% at all tested GA_3 concentrations (Fig. 1a), while the regeneration frequencies in line 238-6-3-1 were slightly lower, up to 94.88% at concentration of 5 µM (Fig. 2a). Generally, GA_3 provoked higher regeneration responses of the explants than GA_1, in both lines (Figs. 1 and 2). On GA_1-supplemented media, the frequency of regeneration did not exceed 86% for either line (Figs. 1a and 2a).

In both lines the highest SE mean number was recorded under LD in the GA_3-treated explants (40.73 and 16.67 for lines 125 and 238-6-3-1, respectively). However, each line required different levels of GA_3 for highest SE-regeneration response, 2.5 µM for line 125 and 5 µM for line 238-6-3-1 (Figs. 1b and 2b), although the differences among the treatments were not statistically significant.

Taken together, SE regeneration was the most efficient in root apices cultivated on media supplemented with 2.5–5 µM GA_3 under LD conditions (Figs. 1 and 2). All SEs obtained in the present study passed through the typical stages of development, converted into plants, flowered, self fertilized, and set seeds in vitro (Supplementary Fig. 1).

Histological analysis

Histological analysis was conducted in order to explore the origin of SEs initiated from the root apices of spinach roots. During the SE-induction treatment (BM + 20 µM NAA + 5 µM GA_3 under LD), the histological alterations were detected in the vascular cylinder and surrounding parenchyma (Fig. 3a and b). The first events leading to SE formation were the periclinal and anticlinal cell divisions in the pericycle and parenchyma tissue around the vascular elements (Fig. 3b). After the initial divisions, cell proliferation occurred, resulting in the establishment of a proliferation zone composed of several cell layers. Small meristematic cells with dense cytoplasm, large nuclei and prominent nucleoli were surrounded by large, vacuolated non-embryonic cells (Fig. 3c). Intensive cell divisions led to the disruption of the well-arranged layers and the formation of irregular proembryonal tissue (Fig. 3d). The transition of proembryoids to globular stage SEs was characterized by the formation of meristematic centers with intense mitotic activity. The proembryonal tissue differentiated into SEs with typical meristematic cells (Fig. 3e and f). Therefore, SEs obtained in the present study originated from the pericycle and parenchyma associated with vascular tissue.

Expression of the GA-ox genes in the root apices

In order to elucidate how light and GA_3 influence the expression of genes encoding the key enzymes for GA metabolism, the expression levels of the *GA-ox* genes

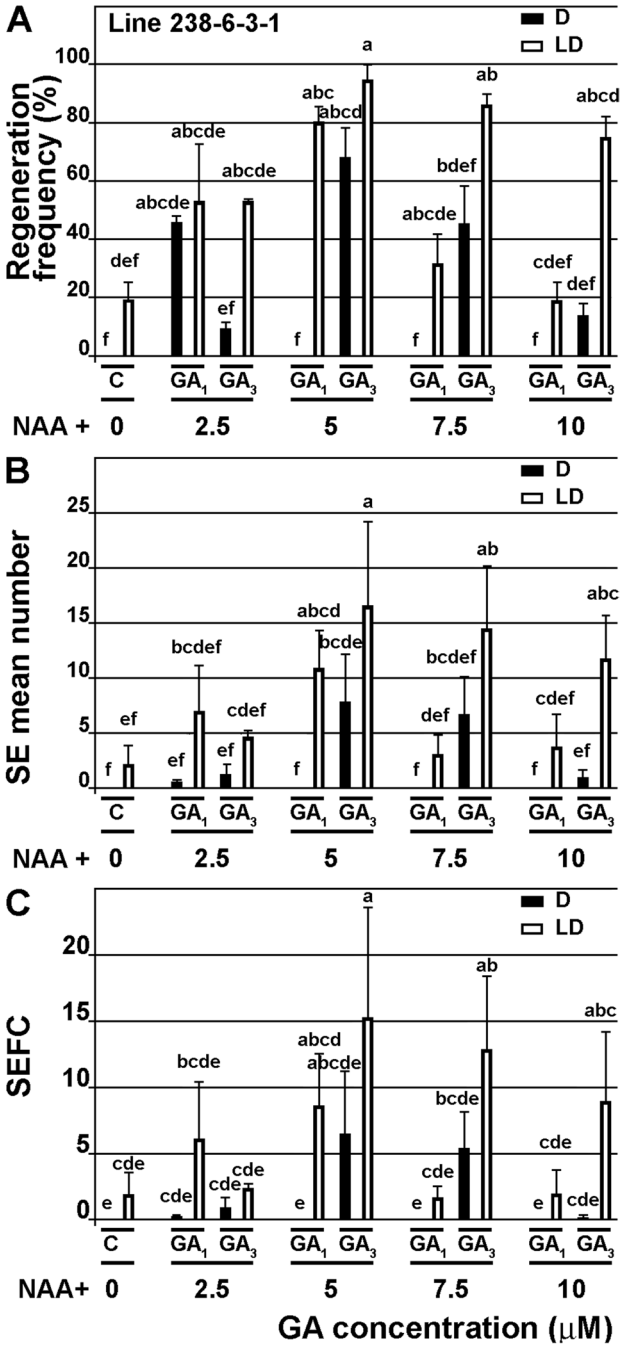

Fig. 2 Somatic embryogenesis from in vitro root apices isolated from SE-derived plants of line 238-6-3-1. The root explants were cultivated on BM supplemented with 20 µM NAA as a sole PGR (control—C) or in combination with 2.5, 5, 7.5, 10 µM GA_1 or GA_3 under long day (LD) or dark (D) conditions for 12 weeks. **a** Frequency of regeneration. **b** The mean SE number per explant. **c** Somatic embryoforming capacity (SEFC). Data represent the mean values. For each treatment, four Petri dishes, each with 5 root apices (n = 20), were used. The values marked with the same letters are not statistically significantly different ($p \leq 0.05$) according to the LSD test

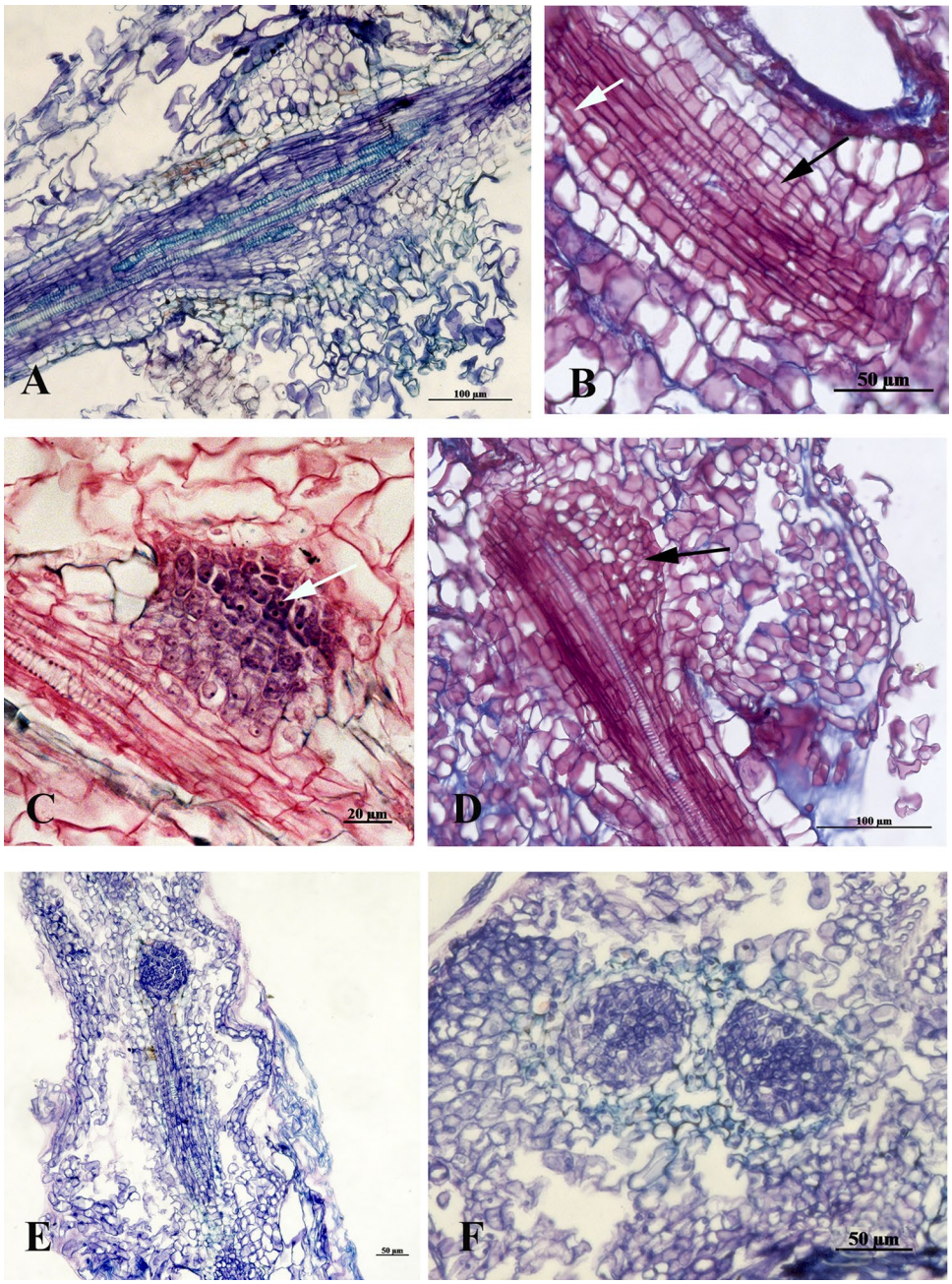

Fig. 3 Histology of somatic embryogenesis from in vitro root apices of spinach cultivated on BM with 20 µM NAA + 5 µM GA_3 under LD conditions for 4 weeks. **a** Cross section of spinach root explant. **b** Periclinal cell divisions were detected in the pericycle (arrows). **c** Cellular proliferation around the vascular cylinder. Note: isodiametric meristemic cells with dense cytoplasm large nuclei and prominent nucleoli (arrow). **d** Cluster of mitotically active cells-proembryo zone arrow. **e** Cross section of the root explant showing a somatic embryo developed from the vascular cylinder. **f** Globular somatic embryo at the periphery of the root explant

(*SoGA20-ox1*, *SoGA3-ox1*, *SoGA2-ox1*, *SoGA2-ox2* and *SoGA2-ox3*) were analyzed. GA_3 was chosen because it had stronger effect on SE induction than GA_1. Amplification efficiency of all *GA-ox* genes was satisfactory (108.8%, 97.2%, 94.9%, 114.7% and 112% for *SoGA20-ox1*, *SoGA3-ox1*, *SoGA2-ox1*, *SoGA2-ox2* and *SoGA2-ox3* respectively), and uniformity of the dissociation curves of the obtained products confirmed the specificity of the amplification reactions for all genes.

To test how light influences the expression of *GA-ox* genes in the absence of GA_3, their expression was measured in root apices cultivated on PGR-free medium under LD conditions and compared to the control ones cultivated in darkness. The expression levels of *SoGA20-ox1*, *SoGA3-ox1*, *SoGA2-ox1*, *SoGA2-ox2* and *SoGA2-ox3* were significantly higher (0.93, 2.39, 2.55, 3.32, and 3.94 relative expression value—REV, respectively) in the explants cultivated under LD compared to those cultivated in the darkness (Fig. 4).

In order to explore coupled influence of GA_3 and light on the expression of *GA-ox* genes, the expression levels of these genes were measured in the root apices cultivated on induction medium containing 20 µM NAA and 5 µM GA_3 under LD for 4 weeks, while the explants cultivated on PGR-free

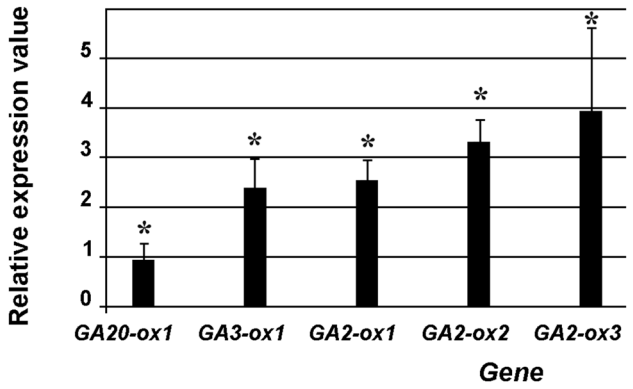

Fig. 4 The expression levels of *SoGA20-ox1*, *SoGA3-ox1*, *SoGA2-ox1*, *SoGA2-ox2 and SoGA2-ox3* in root apices of randomly chosen seedlings grown on PGR-free BM under LD conditions for 4 weeks. The expression was normalized to the expression of the α-tubulin gene, and calculated relative to the expression of the five *GA-ox* genes in the control explants grown in darkness, using ΔΔCt method. The obtained values were subjected to $\log_2$ transformation and presented as relative expression values (REV). Data represent mean values of three independent biological samples, each with three technical repetitions. Values marked with asterisk are significantly different ($p \leq 0.05$) from the control according to t-test for dependent samples

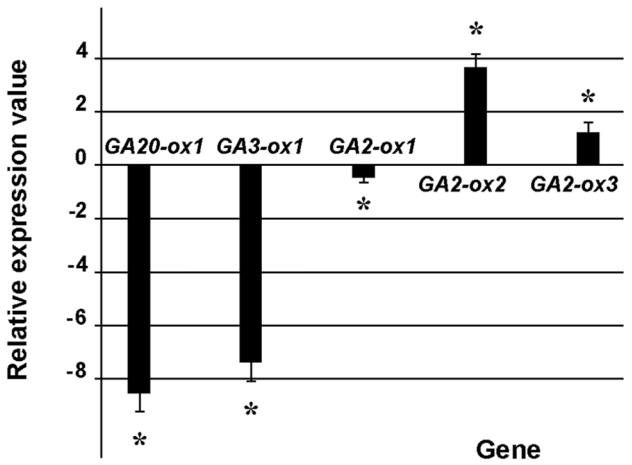

Fig. 5 The expression levels of *SoGA20-ox1*, *SoGA3-ox1*, *SoGA2-ox1, SoGA2-ox2 and SoGA2-ox3* in root apices of randomly chosen seedlings grown on somatic embryo induction medium (20 µM NAA + 5 µM GA_3) under LD conditions for 4 weeks. The expression is normalized to the expression of the α-tubulin gene, and calculated relative to the expression in the control explants grown on PGR-free BM under LD conditions using ΔΔCt method. The obtained values were subjected to $\log_2$ transformation and presented as relative expression values (REV). Data represent mean values of three independent biological samples, each with 3 technical repetitions. Values marked with asterisk are significantly different ($p \leq 0.05$) from the control according to t-test for dependent samples

medium under LD were used as a control. In the explants cultivated on SE induction medium, considerable changes in the expression of *GA-ox* genes were observed (Fig. 5). The expression of both *SoGA20-ox1* and *SoGA3-ox1* significantly decreased (8.56 and 7.41 REV, respectively), the expression of *SoGA2-ox1* decreased only slightly (0.48 REV), while the expression of *SoGA2ox-2* and *SoGA2ox-3* genes significantly increased (3.69 and 1.23 REV, respectively, Fig. 5).

Discussion

Influence of exogenous GAs on SE regeneration from the root sections

The regeneration response of spinach roots is under strong genetic control and exhibits high individual variability (Al-Khayri et al. 1991; Ishizaki et al. 2001; Milojević et al. 2011, 2012; Milić et al. 2017), with differences among the seed-derived lines frequently higher than those observed among the treatments (Milojević et al. 2012). Therefore, the comparisons among the treatments in the present study were conducted on two previously tested single seed-derived lines.

In the majority of dicotyledonous plant species, the exogenous application of auxins is still one of the most commonly used methods to induce the transition of somatic cells into embryogenic ones (Méndez-Hernández et al. 2019). In this respect, auxins can operate directly, but can also provoke the production of endogenous indole-3-acetic acid (IAA) or act as a stress factor (Fehér et al. 2003). However, the results obtained in the present study showed that application of NAA singly was insufficient for satisfactory somatic embryogenesis response from root apices of spinach, even in lines that are genetically predisposed to this process, as are 125 and 238-6-3-1. The results of the present study showed GA was essential for induction of somatic embryogenesis in spinach and underlined strong synergistic effect of light and GA on SE initiation. In the absence of both GA and light, the explants of only one line, line 125, regenerated SEs with extremely low regeneration capacity, while the presence of either factor slightly increased the regeneration responses (Figs. 1 and 2). Therefore, both light and GA are indispensable for highly efficient SE regeneration, as they could not substitute each other for efficient SE induction. This conclusion is consistent with the results obtained for both lines tested in the present study, as well as for a number of different lines used in other studies carried out by our research group.

Synergism of light and GA in promotion of somatic embryogenesis in spinach has not been reported so far, despite GA_3 has been found essential for efficient induction of somatic embryogenesis from root apices of spinach (Komai et al. 1996a, b; Knoll et al. 1997; Ishizaki et al. 2001; Nguyen et al. 2013). Interaction of light and GA on de novo regeneration in spinach has only been studied by

Geekiyanage et al. (2006), who found bud regeneration from the cotyledons to be more efficient under SD than under LD conditions. The discrepancies between the present study and the study of Geekiyanage et al. (2006) may be due to different light requirements of the root apices and cotyledons for induction of de novo regeneration. However, the lack of statistical differences among the treatments in the study of (Geekiyanage et al. 2006) indicates that this may be due to a high genetic variability of plant material used in the study. Reports on interaction of light and GA on SE regeneration in other plant species are scarce. A rare example is SE regeneration from petioles of *Foeniculum vulgare* in which GA_3 and light acted antagonistically; light inhibited SE regeneration and GA_3 abolished its inhibitory effect (Hunault and Maatar 1995).

In the present study, line 125 was superior over the line 238-6-3-1; the SEFC indices were 40.7 vs. 15.3, respectively (Figs. 1c and 2c). The line 238-6-3-1 had the highest SE-forming capacity ever achieved in any of our studies (Milojević et al. 2011), but its regeneration capacity declined over time, as it was maintained for more than 10 years through repetitive cycles of somatic embryogenesis. Although significantly lower than before, SE-forming capacity of this line was still stable and relatively high at the time the present study was undertaken.

The in vitro root apices of lines 125 and 238-6-3-1 were capable of SE regeneration under suboptimal conditions, in the absence of GA_3, unlike other lines tested in a preliminary study. Under more stringent conditions, in the absence of both light and GA_3, the root apices of line 125 were capable of SE initiation, whereas those of line 238-6-3-1 were not. Moreover, the root apices of line 125 required lower levels of GA_3 for efficient initiation of SEs than those of line 238-6-3-1. Therefore, the greater the SE regeneration capacity of a line, the lower requirements for the induction of SE regeneration.

GAs have only seldom been reported to be indispensable for SE initiation, e.g. in: *Theobroma cacao* (Kononowicz and Janick 1984), *Rumex acetosella* (Ćulafić et al. 1987), *Medicago sativa* (Rudus et al. 2002) and *Tylophora indica* (Thomas 2006). In this respect, spinach is quite an exception, as it seems that there are only few examples in the literature where the presence of GA is so important for efficient SE initiation. Such an example is *Foeniculum vulgare*, in which exogenous GA_3 not only increased the efficiency of somatic embryogenesis, but also promoted further development of SEs (Hunault and Maatar 1995). In geranium and *Medicago*, paclobutrazol, an inhibitor of GA biosynthesis, inhibited SE regeneration (Rudus et al. 2002). More often, exogenous GAs had no effect or even had an inhibitory effect on SE initiation, as in *Oncidium* sp. (Chen and Chang 2003), *Arabidopsis thaliana* (Wang et al. 2004), and *Centaurium erythraea* (Subotić et al. 2009).

In the present study, GA_3 showed more pronounced impact on SE initiation than GA_1. Plants are able to synthesize both GA_3 and GA_1, although the former in very small amounts and only under specific conditions (Silva et al. 2013). However, GA_3 was detected in various organs of numerous plant species, e.g. in seeds of *A. thaliana*, *Ipomoea batatas* and *Cucumis sativus*, shoot apices of *Althaea rosea*, shoots of *Lactuca sativa* and *Zea mays*, stems of *Brassica napus*, fruits of *Citrus sinensis*, roots of *Lycopersicon esculentum*, and leaves and roots of *Triticum aestivum* (MacMillan 2001), but the detection of GA_3 in spinach has not been reported so far. Also, to the best of our knowledge, no difference between GA_1 and GA_3 intake efficiency has been reported for any plant species to date. GA transporters (NITRATE TRANSPORTER1/PEPTIDE TRANSPORTER—NPF3) from the root cell membranes of *A. thaliana* are equally able to import both active (GA_3, GA_4, GA_1) and intermediate (GA_8, GA_{19}, GA_{20}) GAs across the membranes (Tal et al. 2016; Binenbaum et al. 2018).

Histological analysis

Histological analysis conducted in the present study revealed multicellular origin of SEs derived from the pericycle cells of spinach roots, where many dividing clusters of meristematic cells were observed (Fig. 3). The root's pericycle cells generally have a remarkable capacity for regeneration of both SEs (Yumbla-Orbes et al. 2017; Yang et al. 2010) and shoot/root primordia (Atta et al. 2009; Rocha et al. 2012; Jani et al. 2015). Dedifferentiation of root pericycle cells and their transition into either proembryonic tissue or meristemoids may result in the formation of SEs or adventitious buds, however, the initial stages of both processes are the same (Yumbla-Orbes et al. 2017). During the initial stages of regeneration, we observed periclinal cell divisions in the pericycle of the root. The first sign of cell reprogramming and modifications in the development process is a change in the direction of cell divisions (Kurczyńska et al. 2007). Periclinal, asymmetric divisions indicate a change in development and have been observed in many species during the development of SEs: *Trifolium repens* (Maheswaran and Williams 1985), *Juglans regia* (Polito et al. 1989) and *Helianthus annuus* × *H. tuberossus* (Chiappetta et al. 2009). Some other characteristics of the cells also point to their further development and fate. The cells which developed into embryo cells are meristematic cells with dense cytoplasm, small vacuoles, and a large nucleus. However, not every cell with meristematic characteristics will develop into an embryonic cell. According to Verdeil et al. (2007), the shape and structure of the nucleus may indicate the further fate of the cells.

Interestingly, some studies found different origin of primordia regenerated from spinach root apices using the same

PGRs. Komai et al. (1996a), who used 10 µM NAA + 0.1 µM GA_3 to induce SEs from spinach roots, obtained massive calli with SEs formed on their surface. However, Knoll et al. (1997), demonstrated direct shoot regeneration from epidermal and subepidermal cells, without a callus phase, from root apices cultivated on medium supplemented with 20 µM NAA + 5 µM GA_3.

Expression of the GA-ox genes in the root explants

Expression of the *GA-ox* genes has been previously analyzed in the above-ground organs of spinach plants (apical bud, young leaves, stems, petioles, male and female flowers), but not in the roots (Wu et al. 1996; Lee and Zeevaart 2002, 2005, 2007). In the present study, the expression of all five analyzed *GA-ox* genes was detected in the root apical fragments, indicating that isolated roots are capable of both perceiving the light and autonomously synthesizing GAs. Root sections in the present study were sampled 4 h after the light was switched on, since the level of *GA20-ox* expression exhibits diurnal variation (Jackson et al. 2000; Hisamatsu et al. 2005), and usually peaks 3–4 h from the onset of light exposure (Ait-Ali et al. 1999; Hisamatsu et al. 2005; Paparelli et al. 2013). Intact roots have the ability to perceive light, regardless of being underground organs, since they express the photoreceptors (Galen et al. 2007; Mo et al. 2015). Also, phytochrome from the shoot can modulate some physiological processes in the roots (Salisbury et al. 2007). However, to the best of our knowledge, nothing is known about isolated roots of spinach.

In the present study, the expression of *SoGA20-ox1* was low in the root segments grown in the darkness on PGR-free medium. A similar, low level expression of *AtGA20-ox4* was also found in the roots of intact *Arabidopsis* plants (Rieu et al. 2008). Exposure to light caused an expected increase in expression of *SoGA20-ox1* in the present study (Fig. 4), since the expression of the *GA20-ox* gene is under photoperiodic control, not only in spinach (Wu et al. 1996; Lee and Zeevaart 2002, 2005, 2007), but also in other plant species (Ait-Ali et al. 1999; Jackson et al. 2000; Hisamatsu et al. 2005). The expression levels of the remaining four *GA-ox* genes in the spinach root apices were also significantly higher in the explants grown under LD conditions than in those grown in darkness. This result is in line with the notion that *GA3-ox* and *GA2-ox* genes are not under photoperiodic control, but that their expression levels rather depend on the substrate (GA_{20}) availability (Hisamatsu et al. 2005). Their higher expression levels can be explained as a result of higher level of *SoGA20-ox* expression.

Subsequently the expression of the *GA-ox* genes was compared between the explants cultivated on SE induction medium (NAA + GA_3) and those cultivated on noninductive PGR-free medium, both grown under LD conditions. Under inductive conditions, a decrease in the expression of both *SoGA20-ox1* and *SoGA3-ox1* was observed (Fig. 5). A decrease in the *SoGA20-ox* expression is probably caused by a negative feedback due to the presence of GA_3 in the medium (Israelsson et al. 2004; Radi et al. 2006). An increase in the expression of *SoGA2-ox2* and *SoGA2-ox3* was probably also provoked by the presence of GA_3 in the medium. Similarly, exogenous GA_4 and GA_3 induced the expression of *AtGA2-ox6* in *Arabidopsis* roots (Wang et al. 2004). Apart from this, exogenous auxin can also modulate the transcript levels of GA biosynthesis and deactivation genes. Although Weston et al. (2009) indicated that endogenous IAA acts to promote GA synthesis and inhibits GA deactivation in the roots of pea, they also concluded that supra-optimal levels of exogenous auxin had the reverse effect elevating the expression of *GA2-ox* genes and thus reducing the endogenous level of bioactive GA in roots. Weston et al. (2009) pointed out a very strong inhibition of root growth in just 24 h, even at 1 µM IAA, resulting in a 75% root growth reduction. Previously, Frigerio et al. (2006) also showed an increase in mRNA expression levels of *GA2-ox* gene after treatment with a high and presumably supra-optimal concentration of exogenous auxin (50 µM NAA) in *Arabidopsis* seedlings.

A relation between the expression of embryogenic capacity and the expression of *GA-ox* genes was also observed in other plant species, but literature data concerning this issue are still fuzzy, without a clear picture of the role of GAs in this process. In carrot, the expression levels of *DsGA20-ox* and *DsGA2-ox* were not altered significantly during SE initiation, while the expression levels of the three *GA3-ox* genes were significantly increased (Mitsuhashi et al. 2003). Similar results were obtained in *Medicago truncatula*, in which the differences in the expression of *MtGA3-ox1* and *MtGA3-ox2* were found between regenerating and non-regenerating lines (Igielski and Kępczyńska 2017). However, in *M. truncatula* exogenously supplied GAs were not necessary for the induction of somatic embryogenesis (Igielski and Kępczyńska 2017).

In *Arabidopsis*, expression of *AtGA2-ox6* was essential for the ability of 35S:AGL15 to promote somatic embryogenesis (Wang et al. 2004). Transformants overexpressing 35S:*AtGA2-ox6* produced SE with higher efficacy, while ga2-ox mutants had far smaller embryogenic potential (Wang et al. 2004). All these findings support the hypothesis that SE initiation in some plant species, including spinach, is favored by decreased expression levels of GA anabolic genes and increased expression levels of GA catabolic genes, but this idea requires further research. Considering the additional evidence of the reciprocal interaction between GA and auxin, further research should undoubtedly include gene expression occurrences related to auxin-GA cross-talk during SE induction treatment. Namely, the results of Li

et al. (2015) showed that exogenous GA can modulate auxin signaling and transport, and thus enhance the responsiveness of *Arabidopsis* roots to exogenous auxin. Since that earlier reports indicated auxins, including NAA, to be an important factor of SE-response in many plant species by conferring embryogenic competence of the explant cells, the comprehensive research in the field of interaction between GA and auxin, alongside with already evidenced synergistic effect of light and GA, is needed in order to elucidate the major events that promote the acquisition of embryo competence in spinach.

Conclusions

The present study showed a strong and synergistic effect of light and GAs on SEs initiation from the in vitro root apices of spinach. Both factors are indispensable for the efficient SE regeneration from the explants, and neither can substitute for the other. In addition, the lines with higher SE-forming capacity are capable of SE regeneration under suboptimal conditions. Histological study revealed that SEs originated from the roots' pericycle cells. Expression analysis revealed decreased expression levels of genes encoding GA anabolic enzymes and increased expression levels of genes encoding GA catabolic enzymes, indicating that lower levels of GA may favor SE initiation. This system for SE regeneration from spinach roots is particularly interesting for understanding the role of GA in SE initiation, since GAs were only seldom reported to directly influence this process. Since auxin was also included in SE induction media, the interaction between GA and auxin merit particular attention in further attempts for understanding the SE response in spinach. Nevertheless, the results of the present work open a new window into elucidation of mechanism that underlies SE induction in spinach, involving auxins, GAs and light. A better understanding of these mechanisms may allow us to postulate the conditions that would enable further optimization of somatic embryogenesis in this plant species, which is still considered rather recalcitrant to in vitro regeneration.

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Author contributions JM and SZK designed the research. MB, ST, NB and JM conducted tissue culture experiments. MB and DJ conducted histological study, and MB, JS and JM performed RTqPCR analysis. MB and JM analyzed data. JM, SZK, DJ and MB wrote the manuscript and JS, ST and NB contributed to editing. All authors read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

Ait-Ali T, Frances S, Weller JL, Reid JB, Kendrick RE, Kamiya Y (1999) Regulation of gibberellin 20-oxidase and gibberellin 3β-hydroxylase transcript accumulation during de-etiolation of pea seedlings. Plant Physiol 121:783–791

Al-Khayri JM, Huang FH, Morelock TE, Busharar TA, Gbur EE (1991) Genotype-dependent response of spinach cultivars to *in vitro* callus induction and plant regeneration. Plant Sci 78:121–126

Atta R, Laurens L, Boucheron-Dubuisson E, Guivarc'h A, Carnero E, Giraudat-Pautot V, Rech P, Chriqui D (2009) Pluripotency of *Arabidopsis* xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown in vitro. Plant J 57:626–644

Binenbaum J, Weinstain R, Shani E (2018) Gibberellin localization and transport in plants. Trends Plant Sci 23:410–421

Blazquez S, Olmos E, Hernández JA, Fernández-García N, Fernández JA, Piqueras A (2009) Somatic embryogenesis in saffron (*Crocus sativus* L.). Histological differentiation and implication of some components of the antioxidant enzymatic system. Plant Cell Tissue Org Cult 97:49–57

Cabrera-Ponce JL, López L, León-Ramírez CG, Jofre-Garfias AE, Verver-y-Vargas A (2015) Stress induced acquisition of somatic embryogenesis in common bean *Phaseolus vulgaris* L. Protoplasma 252:559–570

Chen JT, Chang WC (2003) Effects of GA, ancymidol, cycocel and paclobutrazol on direct somatic embryogenesis of *Oncidium in vitro*. Plant Cell Tissue Org Cult 72:105–108

Chiappetta A, Fambrini M, Petrarulo M, Rapparini F, Michelotti V, Bruno L, Greco M, Baraldi R, Salvini M, Pugliesi C, Bitonti MB (2009) Ectopic expression of *LEAFY COTYLEDON1-LIKE* gene and localized auxin accumulation mark embryogenic competence in epiphyllous plants of *Helianthus annuus* × *H. tuberosus*.. Ann Bot 103:735–747

Ćulafić LJ, Budimir S, Vujičić R, Nešković M (1987) Induction of somatic embryogenesis and embryo development in *Rumex acetosella* L. Plant Cell Tissue Org Cult 11:133–139

Economou AS, Read PE (1987) Light treatments to improve efficiency of *in vitro* propagation systems. Hortic Sci 22:751–754

Fehér A, Pasternak TP, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Org Cult 74:201–228

Frigerio M, Alabadí D, Pérez-Gómez J, García-Cárcel L, Phillips AL, Hedden P, Blázquez MA (2006) Transcriptional regulation of gibberellin metabolism genes by auxin signaling in *Arabidopsis*. Plant Physiol 142:553–563

Galen C, Rabenold JJ, Liscum E (2007) Light-sensing in roots. Plant Signal Behav 2:106–108

Gašić K, Hernandez A, Korban S (2004) RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. Plant Mol Biol Rep 22:437–438

Geekiyanage S, Takase T, Watanabe S, Fukai S, Kiyouse T (2006) The combined effect of photoperiod, light intensity and GA_3 on adventitious shoot regeneration from cotyledons of spinach (*Spinacia oleracea* L.). Plant Biotechnol 23:431–435

Graebe JE (1987) Gibberellin biosynthesis and control. Annu Rev Plant Physiol 38:419–465

Hedden P, Phillips AL (2000) Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci 5:523–530

Hisamatsu T, King RW, Helliwell CA, Koshioka M (2005) The involvement of gibberellin 20-oxidase genes in phytochrome regulated petiole elongation of *Arabidopsis*. Plant Physiol 138:1106–1116

Hunault G, Maatar A (1995) Enhancement of somatic embryogenesis frequency by gibberellic acid in fennel. Plant Cell Tissue Org Cult 41:171–176

Igielski R, Kępczyńska E (2017) Gene expression and matabolite profiling of gibberellin biosynthesis during induction of somatic embryogenesis in *Medicago truncatula* Gaertn. PLoS ONE 12:e0182055. https://doi.org/10.1371/journal.pone.0182055

Ishizaki T, Komai F, Masuda K (2001) Screening for strongly regenerative genotypes of spinach in tissue culture using subcultured root explants. Plant Cell Tissue Org Cult 67:251–255

Israelsson M, Mellerowcz E, Chon M, Gullberg J, Moritz T (2004) Cloning and overproduction of gibberellin 3-oxidase in hybrid aspen trees. Effect on gibberellin homeostasis and development. Plant Physiol 135:221–230

Jackson SD, James PE, Carrera E, Prat S, Thomas B (2000) Regulation of transcript levels of a potato gibberellin 20-oxidase gene by light and phytochrome B. Plant Physiol 124:423–430

Jani JN, Jha SK, Nagar DS (2015) Root explant produces multiple shoot from pericycle in *Psoralea corylifolia*—a leprosy destroyer medicinal plant. Ind Crops Prod 67:324–329

Joseph JA, Shukitt-Hale B, Denisova NA, Bielinski D, Martin A, McEwen JJ, Bickford PC (1999) Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. J Neurosci 19:8114–8121

Kanwar K, Joseph J, Deepika R (2010) Comparison of *in vitro* regeneration pathways in *Punica granatum* L. Plant Cell Tissue Org Cult 100:199–207

Kawade K, Masuda K (2009) Transcriptional control of two ribosome – inactivating protein genes expressed in spinach (*Spinacia oleracea*) embryos. Plant Physiol Biochem 47:327–334

Kim DH, Lee B, Kim MJ, Park MH, An HJ, Lee EK, Chung KW, Park JW, Yu BP, Choi JS, Chung HY (2016) Molecular mechanism of betaine on hepatic lipid metabolism: inhibition of forkhead box O1 (FoxO1) binding to peroxisome proliferator-activated receptor gamma (PPARγ). J Agric Food Chem 64:6819–6825

Knoll KA, Short KC, Curtis IS, Power JB, Davey JB (1997) Shoot regeneration from cultured root explants of spinach (*Spinacia oleracea* L.): a system for *Agrobacterium* transformation. Plant Cell Rep 17:96–101

Komai F, Okwe I, Harada T (1996a) Somatic embryogenesis and plant regeneration in culture of root segments of spinach (*Spinacia oleracea L.*). Plant Sci 113:203–208

Komai F, Okuse I, Harada T (1996b) Effective combinations of plant growth regulators for somatic embryogenesis from spinach root segments. J Jpn Soc Hortic Sci 65:559–564

Kononowicz H, Janick J (1984) Response of embryogenic callus of *Theobroma cacao* L. to gibberellic acid and inhibitors of gibberellic acid synthesis. Z Pflanzenphysiol 113:359–366

Kurczyńska EU, Gaj MD, Ujczak A, Mazur E (2007) Histological analysis of direct somatic embryogenesis in *Arabidopsis thaliana* (L.) Heynh. Planta 226:619–628

Lee DJ, Zeevaart JAD (2002) Differential regulation of RNA levels of gibberellin dioxygenases by photoperiod in spinach. Plant Physiol 130:2085–2094

Lee DJ, Zeevaart JAD (2005) Molecular cloning of GA 2-oxidase3 from spinach and its ectopic expression in *Nicotiana sylvestris*. Plant Physiol 138:243–254

Lee DJ, Zeevaart JAD (2007) Regulation of gibberellin 20-oxidase1 expression in spinach by photoperiod. Planta 226:35–44

Li G, Zhu C, Gan L, Ng D, Xia K (2015) GA_3 enhances root responsiveness to exogenous IAA by modulating auxin transport and signalling in *Arabidopsis*. Plant Cell Rep 34:483–494

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time quantitative PCR and the ΔΔCt method. Methods 25:402–408

Lomnitski L, Bergman M, Nyska A, Ben-Shaul V, Grossman S (2003) Composition, efficacy, and safety of spinach extracts. Nutr Cancer 46:222–231

MacMillan J (2001) Occurence of gibberellins in vascular plants, fungi and bacteria. J Plant Growth Regul 20:387–442

Maheswaran G, Williams EG (1985) Origin and development of somatic embryoids formed directly on immature embryos of *Trifoliumrepens in vitro*. Ann Bot 56:619–630

Mc Manus JFA, Mowry RW (1960) Staining methods. In: Hoeber PB (ed) Histology and histochemistry. Harper and Row, New York, pp 275–276

Méndez-Hernández HA, Ledezma-Rodríguez M, Avilez-Montalvo RN, Juárez-Gómez YL, Skeete A, Avilez-Montalvo J, De-la-Peña C, Loyola-Vargas VM (2019) Signaling overview of plant somatic embryogenesis. Front Plant Sci 10:77. https://doi.org/10.3389/fpls.2019.00077

Milić M, Savić J, Tubić LJ, Devrnja N, Ćalić D, Zdravković-Korać S, Milojević J (2017) Expression of the gene for ribosome-inactivating protein, *SoRIP2*, as a tool for the evaluation of somatic embryogenesis in spinach. Plant Cell Tissue Org Cult 129:483–491

Milojević J, Tubić Lj, Zdravković-Korać S, Dragićević I, Ćalić-Dragosavac D, Vinterhalter B (2011) Increased regeneration capacity in spinach lines obtained by *in vitro* self-fertilisation. Sci Hortic 130:681–690

Milojević J, Tubić L, Pavlović S, Mitić N, Ćalić D, Vinterhalter B, Zdravković-Korać S (2012) Long days promote somatic embryogenesis in spinach. Sci Hortic 142:32–37

Mitsuhashi W, Toyomasu T, Masui H, Katho T, Nakaminami K, Kashiwagi Y, Akutsu M, Kenmoku H, Sassa T, Yamaguchi S, Kamiya Y, Kamada H (2003) Gibberellin is essentially required for carrot (*Daucus carota* L.) somatic embryogenesis: dynamic regulation of gibberellin 3-oxidase gene expressions. Biosci Biotechnol Biochem 67:2438–2447

Mo M, Yokawa K, Wan Y, Baluška F (2015) How and why do root apices sense light under the soil surface? Front Plant Sci 6:775. doi:https://doi.org/10.3389/fpls.2015.00775

Muktadir MA, Habib MA, Milan MAK, Akhond MAY (2016) Regeneration efficiency based on genotype, culture condition and growth regulators of eggplant (*Solanum melongena* L.). Agric Nat Resour 50:38–42

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Nameth B, Dinka SJ, Chatfield SP, Morris A, English J, Lewis D, Oro R, Raizada MN (2013) The shoot regeneration capacity of excised *Arabidopsis* cotyledons is established during the initial hours after injury and is modulated by a complex genetic network of light signalling. Plant Cell Environ 36:68–86

Nguyen QV, Sun HJ, Boo KH, Lee D, Lee JH, Lim PO, Lee HY, Riu KZ, Lee DS (2013) Effect of plant growth regulator combination and culture period on in vitro regeneration of spinach (*Spinacia oleracea* L.). Plant Biotechnol Rep 7:99–108

Paparelli E, Parlanti S, Gonzali S, Novi G, Mariotti L, Ceccarelli N, van Dongen JT, Kölling K, Zeeman SC, Perata P (2013) Nighttime sugar starvation orchestrates gibberellin biosynthesis and plant growth in *Arabidopsis*. Plant Cell 25:3760–3769

Polito VS, McGranahan G, Pinney K, Leslie C (1989) Origin of somatic embryos from repetitively embryogenic cultures of walnut (*Juglans regia* L.): implications for *Agrobacterium* mediated transformation. Plant Cell Rep 8:219–221

Radi A, Lange T, Niki T, Koshioka M, Pimenta Lange MJ (2006) Ectopic expression of pumpkin gibberellin oxidases alters gibberellin biosynthesis and development of transgenic *Arabidopsis* plants. Plant Physiol 140:528–536

Reuveni M, Evenor D (2007) On the effect of light on shoot regeneration in petunia. Plant Cell Tissue Org Cult 89:49–54

Rieu I, Ruiz-Rivero O, Fernandez-Garcia N, Griffiths J, Powers SJ, Gong F, Linhartova T, Eriksson S, Nilsson O, Thomas SG, Phillips AL, Hedden P (2008) The gibberellin biosynthetic genes AtGA20ox1 and AtGA20ox2 act, partially redundantly, to promote growth and development throughout the Arabidopsis life cycle. Plant J 53:488–504

Roberts JL, Moreau R (2016) Functional properties of spinach (*Spinacia oleracea* L.) phytochemicals and bioactives. Food Funct 7:3337–3353

Rocha DI, Vieira LM, Tanaka FAO, da Silva LC, Oton WC (2012) Anatomical and ultrastructural analyses of *in vitro* organogenesis from root explants of commercial passion fruit (*Passiflora edulis* Sims). Plant Cell Tissue Org Cult 111:69–78

Rudus I, Kępczyńska E, Kępczyński J (2002) Regulation of *Medicago sativa* L. somatic embryogenesis by gibberellins. Plant Growth Regul 36:91–95

Sakai WS (1973) Simple method for differential staining of paraffin embedded plant material using toluidine blue. Stain Technol 48:247–249

Salisbury FJ, Hall A, Grierson CS, Halliday KJ (2007) Phytochrome coordinates *Arabidopsis* shoot and root development. Plant J 50:429–438

Silva ALL, Rodriguez C, Costa JL, Machado MP, Penha RO, Buasi LA, Vanderberghe LPS, Soccol CR (2013) Gibberellic acid fermented extract obtained by solid-state fermentation using citric pulp by *Fusarium moniliforme*: influence on *Lavandula anguistifolia* Mill. cultivated *in vitro*. Pak J Bot 45:2057–2064

Shohag MJI, Wei YY, Yu N, Zhang J, Wang K, Patring J, He Z, Yang X (2011) Natural variation of folate content and composition in spinach (*Spinacia oleracea*) germplasm. J Agric Food Chem 59:12520–12526

Subotić A, Jevremović S, Trifunović M, Petrić M, Milošević S, Grubišić D (2009) The influence of gibberellic acid and paclobutrazol on induction of somatic embryogenesis in wild type and hairy root cultures of *Centaurium erythraea* Gillib. Afr J Biotechnol 8:3223–3228

Tal I, Zhang Y, Jørgensen ME, Pisanty O, Barbosa ICR, Zourelidou M, Regnault T, Crocoll C, Olsen CE, Weinstain R, Schwechheimer C, Halkier BA, Nour-Eldin HH, Estelle M, Shani E (2016) The *Arabidopsis* NPF3 is a GA transporter. Nat Commun 7:11486. https://doi.org/10.1038/ncomms11486

Talla ST, Madam E, Manga S, Aileni M, Mamidala P (2018) Efficient TDZ-induced regeneration from capitulum explants of *Gerbera jamesonii* Bolus ex Hooker F.—an ornamental plant with high aesthetic value. Plant Biosyst 153:679–685

Talon M, Zeevaart JAD, Gage DA (1991) Identification of gibberellins in spinach and effects of light and darkness on their levels. Plant Physiol 97:1521–1526

Thomas TD (2006) Effect of sugars, gibberellic acid and abscisic acid on somatic embryogenesis in *Tylophora indica* (Burm. f.) Merrill. Chin J Biotechnol 22:465–471

Verdeil JL, Alemanno L, Niemenak N, Trambarger TJ (2007) Pluripotent versus totipotent plant stem cells: dependence versus autonomy? Trends Plant Sci 12:245–252

Wang H, Caruso LV, Downie AB, Perry SE (2004) The embryo MADS domain protein AGAMOUS-Like 15 directly regulates expression of a gene encoding an enzyme involved in gibberellin metabolism. Plant Cell 16:1206–1219

Weston DE, Reid JB, Ross JJ (2009) Auxin regulation of gibberellin biosynthesis in the roots of pea (*Pisum sativum*). Funct Plant Biol 36:362–369

Wu K, Li L, Gage DA, Zeevaart JAD (1996) Molecular cloning and photoperiod-regulated expression of gibberellin 20-oxidase from the long-day plant spinach. Plant Physiol 110:547–554

Yamaguchi S, Kamiya Y (2000) Gibberellin biosynthesis: its regulation by endogenous and environmental signals. Plant Cell Physiol 3:251–257

Yang JL, Seong ES, Kim MJ, Ghimire BK, Kang WH, Yu CY, Li CH (2010) Direct somatic embryogenesis from pericycle cells of broccoli (*Brassica oleracea* L. var. *italica*) root explants. Plant Cell Tissue Org Cult 100:49–58

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden T (2012) Primer–BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinform 13:134–145

Yumbla-Orbes M, Ferreira da Cruz AC, Marques Pinheiro MV, Rocha DI, Batista DS, Koehler AD, Barbosa JG, Otoni WC (2017) Somatic embryogenesis and *de novo* shoot organogenesis can be altenatively induced by reactivating pericycle cells in Lisianthus (*Eustoma grandifolium* (Raf.) Shinners) root explants. In vitro Cell Dev Biol Plant 53:209–218

Zeevaart JAD, Gage DA, Talon M (1993) Gibberellin A_1 is required for stem elongation in spinach. Proc Natl Acad Sci USA 90:7401–7405